Biochem. J. (1976) **160**, 417–426
Printed in Great Britain

Demonstration of a New Mammalian Isoleucine Catabolic Pathway Yielding an *R* Series of Metabolites

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(*Received* 21 *May* 1976)

1. Normal human urine contains small amounts (less than 4mg/g of creatinine) of 2-ethylhydracrylic acid, formed, we believe, by a previously undisclosed endogenous catabolic pathway for the oxidation of a newly described series of *R* metabolites of isoleucine. 2. Urinary excretion of 2-ethylhydracrylic acid is variably increased in defects of isoleucine oxidation at distal steps in the catabolic pathway (3-oxoacyl-CoA thiolase deficiency and methylmalonyl-CoA mutase deficiency) and is diminished when proximal steps of the oxidative pathway are blocked as in branched-chain oxo acid decarboxylase deficiency ('maple-syrup-urine' disease). 3. Precursors of *R*-pathway metabolites [*R*(−)-2-methylbutyrate and 2-ethylacrylate] lead to increased 2-ethylhydracrylate excretion in the mammal (rat, rabbit and dog); the corresponding *S* metabolites [*S*(+)-2-methylbutyric acid and tiglic acid], when given in equimolar amounts, have little effect on its excretion, suggesting that little or no interconversion between *S* and *R* metabolites occurs *in vivo*. 4. Studies with ^{2}H-labelled precursors indicate that conversion of *R* 2-methylbutyrate into 2-ethylhydracrylic acid occurs by a direct pathway (apparently via 2-ethylacrylic acid). 5. The further oxidation of 2-ethylhydracrylic acid to ethylmalonic acid was demonstrated, and may be analogous to *S*-metabolite oxidation via methylmalonate. 6. Valine metabolites do not interact with the *R*-isoleucine pathway under the conditions of these experiments *in vivo*.

It is generally accepted that L(+)-isoleucine (2*S*, 3*S*) is oxidized by the catabolic pathway originating in *S* 2-oxo-3-methylpentanoic acid and yielding acetyl- and propionyl-CoA by the 3-oxoacyl-CoA thiolase-mediated cleavage of 2-methylacetoacetyl-CoA (Robinson *et al.*, 1956). The inborn error of isoleucine catabolism in man affecting the 3-oxoacyl-CoA thiolase step and leading to the accumulation of 2-methylacetoacetic acid, 2-methyl-3-hydroxybutyric acid and tiglic acid in body fluids (Daum *et al.*, 1971, 1973; Keating *et al.*, 1972; Hillman & Keating, 1974; Gompertz *et al.*, 1974) lends additional support to this pathway. Another metabolite, 2-ethylhydracrylic acid, present in normal urine (1–4mg/g of creatinine), has also been detected (Mamer & Tjoa, 1974) in increased amounts in the urine of patients with deficient β-oxothiolase (EC 2.3.1.16). Further, this metabolite bearing ^{2}H substitution has been found in the urine of rats injected with racemic 2-[*Me*-$^2H_3$]methylbutyric acid (Mamer & Tjoa, 1975). These observations suggested to us the possibility of an alternate minor *R* pathway (Fig. 1) with intermediates derived as a consequence of the chiral nature of the 3-carbon of L(+)-alloisoleucine (2*S*, 3*R*).

Mammals form L-alloisoleucine through re-amination of *R* 2-oxo-3-methylpentanoate produced by the oxo–enolic racemization of the *S* oxo acid product of L-isoleucine transamination (Meister & White, 1951; Scriver & Rosenberg, 1973; Halpern & Pollock, 1970). The possibility that the respective *S* and *R* 2-oxo acids derived from L-isoleucine could have different catabolic fates appears not to have been previously considered in detail. The major consideration up to now has been directed at the re-amination step resulting in the formation of the relatively inert amino acid L-alloisoleucine (Scriver & Rosenberg, 1973) as the initial metabolite of the *R* pathway. Some of the essential features of the *R* alternative pathway were indeed proposed by Stadler (1959), who found significantly increased urinary excretion of ethylmalonic acid after feeding of DL-isoleucine to rats, but

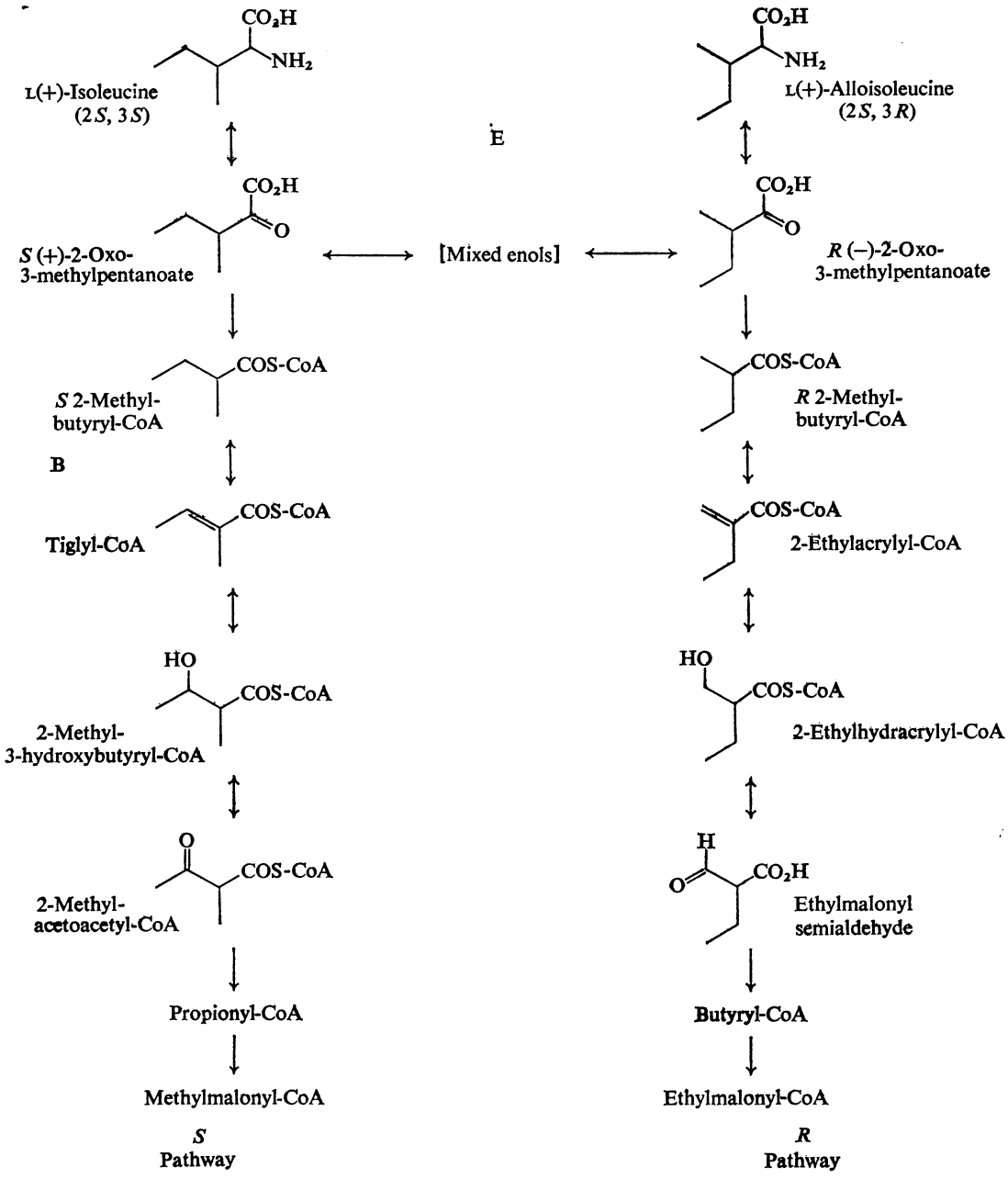

Fig. 1. *Conventional S pathway for isoleucine oxidation (left sequence) and the proposed parallel R pathway for alloisoleucine oxidation beginning at R(−)-2-oxo-3-methylpentanoate (right sequence)*

Termination of the *S* pathway is with succinate in the tricarboxylic acid cycle. 2-Ethylhydracrylic acid undergoes a slow reversible oxidation to presumably the semialdehyde, which is further catabolized to ethylmalonic acid. Although the inclusion of butyryl-CoA in the *R* sequence is not established, it is tentatively included here by analogy with the *S* and valine pathways.

further studies were not pursued. It was the finding of 2-ethylhydracrylic acid in urine together with the failure of L(+)-isoleucine feeding to provoke a significant increase in ethylmalonic acid excretion in the normal human (Mamer & Tjoa, 1975) that led us to renew the investigation of an *R* pathway serving isoleucine oxidation. The present paper reports evidence in support of that hypothesis.

Materials and Methods

Synthesis of intermediates

2-Ethylacrylic acid. The Grignard reagent, prepared in anhydrous tetrahydrofuran from magnesium turnings and 2-bromobut-1-ene, was rapidly carbonated with a large excess of powdered solid CO_2. After acid hydrolysis the mixture was extracted with diethyl ether, evaporated at decreased pressure, and the residue subjected to steam-distillation.

The organic phase was salted out, dried with $MgSO_4$ and distilled at room temperature (25°C) under high vacuum into a bulb at −78°C. A homogeneous product was obtained with a mass spectrum consistent with 2-ethylacrylic acid. The free acid shows a tendency to polymerize and was used the same day for the experiments *in vivo* described below.

2-Methyl[3,3,4,4,4-$^2H_5$]butyric acid. [$^2H_5$]Ethyl iodide (2.6g; Merck, Sharp and Dohme, Montreal, Canada), of 99% minimum isotropic purity, diethyl methylmalonate (3.5g) and ethanolic sodium ethoxide prepared from 0.5g of sodium metal and 8ml of absolute ethanol were refluxed together for 5h in the manner of a standard malonate ester synthesis. The residue obtained from evaporation of the mixture was saponified in 20% KOH by refluxing for 2h. The crude methyl[*Et*-$^2H_5$]ethylmalonic acid was isolated by ether extraction of the acidified saponification mixture and then decarboxylated in 50ml of refluxing 3M-H_2SO_4 for 24h. Steam-distillation yielded 0.75g of product (35% yield) having no impurities as identified by g.l.c.–mass spectrometry of its trimethylsilyl derivative prepared with undiluted trimethylsilylimidazole (Mamer & Gibbs, 1973). Its mass spectrum revealed complete $^2H_5$ substitution (Mamer & Gibbs, 1973) and was analogously consistent with unlabelled 2-methylbutyric acid.

2-[Me-$^2H_3$]Methylbutyric acid. This acid was synthesized by the above method from [$^2H_3$]methyl iodide and diethyl ethylmalonate.

[Et-$^2H_5$]Ethylmalonic acid. This acid was synthesized from dimethyl malonate (0.53g), [$^2H_5$]ethyl iodide (0.50g) and ethanolic sodium ethoxide (0.1g of sodium metal, 10ml of ethanol) as described above. The crude acid extracted after saponification was recrystallized from ethyl acetate (yield 0.30g, 70%). The mass spectra of the expected bis- and tris-trimethylsilyl derivatives (Mamer & Tjoa, 1973) yielded evidence of molecular ions at *m/e* 281 and 353 respectively, and fragmentation routes analogous with the unlabelled ethylmalonic acid derivatives. No unlabelled product was detectable.

Resolution of R and S 2-methylbutyric acid

These resolutions were achieved by fractional crystallization of the quaternary salts formed by the racemic acid with (+)- and (−)-methylbenzylamine (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) by the method of Odham (1963).

Studies in vivo

Studies were performed *in vivo* in the rat, rabbit and dog with the known fatty acid catabolic intermediates of isoleucine and the corresponding intermediates of the proposed *R* pathway.

A female dog (17kg) was anaesthetized with sodium phenobarbital and ventilated under positive pressure. The electrocardiogram was recorded. Cannulae were placed in the femoral vein and the stomach and a catheter in the bladder. A constant urine flow was maintained with 0.9% NaCl infusion and furosemide (5mg intravenously). Control urine and serum samples were obtained before intragastric administration of 10g of racemic 2-methylbutyric acid in 75ml of NaOH at a concentration that maintained solubility (final pH6). Intense salivation, tachycardia and prolongation of Q–T interval on the electrocardiogram occurred in the initial 20min after administration via gastric intubation. Blood gases were kept within the physiological range throughout the study by adequate ventilation and intravenous $NaHCO_3$. At timed intervals the bladder was drained as completely as possible and a 5–10ml sample of urine retained. Blood samples (3ml) were also collected periodically; the serum was removed immediately after clotting. Urine and serum were frozen at −20°C until analysis.

Sprague–Dawley rats (males, 250–300g) were housed in pairs in standard metabolic cages (Canadian Laboratory Supplies, Montreal, Que., Canada) after intraperitoneal injection of 2-ethylacrylic acid (50mg/rat) dissolved in the minimum volume of water and made pH6 with NaOH. The rats were starved for 16h before and during the urine collection (24h) but had free access to water. Similar experiments in rats were conducted with tiglic acid, *S*(+)-2-methylbutyric acid, *R*(−)-2-methylbutyric acid, 2-[*Me*-$^2H_3$]methylbutyric acid and 2-methyl[3,3,4,4,4-$^2H_5$]butyric acid. The urine samples were frozen until analysed.

A female rabbit (2.3kg) was prepared as described for the dog. Isobutyric acid (500mg) was administered by gastric tube to start the experiment, followed by a similar amount of 2-[*Me*-$^2H_3$]methylbutyric acid at 90min. Urine and serum samples were collected at intervals and stored as described above.

Three patients with β-oxothiolase deficiency

(2-methylacetoaceticaciduria), three with branched-chain α-oxo acid decarboxylase deficiency (maple-syrup-urine disease), one with cobalamin-responsive methylmalonicaciduria and six normal subjects provided random samples of urine.

Analysis of urine and serum samples

Organic acids were isolated from serum deproteinized with ethanol (equal vol.) and from urine by extraction into ether and analysed by g.l.c.–mass spectrometry (LKB 9000) as the trimethylsilyl derivatives (Mamer & Gibbs, 1973; Gibbs *et al.*, 1972).

Conditions were as follows: a glass column (2m×6mm) of 3% OV-1 on Chromosorb W HP was programmed to give a temperature rise from 80° to 280°C at 5°C/min (helium flow rate, 35ml/min; injector, 280°C; separator, 280°C; ion source, 290°C, with an ionizing energy of 70eV at 60μA).

Mass spectra and retention times of the peaks eluted were compared with commercially available or synthesized standards.

Results

Excretion of 2-ethylhydracrylic acid by the human subject

The concentration of 2-ethylhydracrylic acid relative to that of creatinine in the urine of normal children, normal adults and patients with several metabolic diseases affecting isoleucine catabolism is shown in Table 1. The identity of the substance as 2-ethylhydracrylic acid originally referred to as an 'ω-hydroxypentanoic acid, unknown isomer' (Mamer & Tjoa, 1974) has now been proven rigorously (Mamer & Tjoa, 1975).

Mammalian response to loading with intermediates of the R and S pathways

2-Ethylhydracrylic acid is below the limit of detectability (0.01 μg/ml) in serum of the normal dog, and comprises a very minor constituent of dog urine (Fig. 2). After administration of racemic 2-methylbutyrate, the concentration of 2-ethylhydracrylic acid in serum rose to 63μg/ml within 120min and urine excretion increased to 7.5g/g of creatinine; the latter is 4000 times the pre-load value. Serum and urine lactate concentrations fell and rose respectively. 3-Hydroxybutyric acid showed no change in serum, but increased about 50-fold in urine. Excretion of *N*-2-methylbutyrylglycine (5mg/g of creatinine) was also observed.

Excretion of organic acids in the urine is influenced in the rat by injection of various presumed fatty acid intermediates of isoleucine oxidation (Table 2). The greatest excretion of the *R* metabolite 2-ethylhydracrylate is precipitated by *R* 2-methylbutyrate and 2-ethylacrylate; conversely, these two acids have little effect on the excretion of the *S*-pathway metabolite 2-methyl-3-hydroxybutyrate.

Table 1. *Urinary excretion of 2-ethylhydracrylic acid in man*

Normal children (5–9 years old) and adults showed no differences by the Wilcoxon rank test in excretion of 2-ethylhydracrylic acid. Details of the isoleucine load are given elsewhere (Mamer & Tjoa, 1974). The low-protein diet is described in Daum *et al.* (1973); patients were in good metabolic control. The untreated newborn patient was acutely ill, and results are before treatment; patient is as described in Daum *et al.* (1973). Each patient with maple-syrup-urine disease had serum isoleucine concentration in excess of 1 mM and high concentrations of the corresponding branched-chain α-oxo acid (2-oxo-3-methylpentanoate). The numbers of observations are given in parentheses.

Subjects	Excretion rate (mg/g of creatinine)
Normal children and adults	1–4 (6)
Normal adult	
Pre-load	2.8 (1)
After isoleucine load (175 mg/kg)	10.4 (1)
Patients with 3-oxo acyl thiolase deficiency	
Low-protein diet	1.6–6.5 (2)
Untreated newborn	63 (1)
Patient with untreated methylmalonicaciduria (vitamin B-12-responsive)	7.4–9.3 (1)
Patients with maple-syrup-urine disease	<0.8 (3)

Response to administration of ^2H-labelled 2-methylbutyric acids

2-[*Me*-$^2H_3$]*Methylbutyric acid in the rabbit.* Administration of the valine metabolite, isobutyric acid (the equivalent of 2-methylbutyrate in the isoleucine pathways) by gastric intubation to the rabbit provoked an abrupt increase in urinary excretion of 3-hydroxyisobutyric acid [the equivalent of 2-methyl-3-hydroxybutyrate and 2-ethylhydracrylic acid in the *S*- and *R*-isoleucine pathways respectively (Fig. 3)]. The concentration of methylmalonic acid increased to 6.8mg/g of creatinine in urine after the loading procedure (not shown on Fig. 3). The excretion of 2-ethylhydracrylic acid decreased slightly under these conditions. Racemic 2-[*Me*-$^2H_3$]methylbutyrate given at 90min in the rabbit experiment caused 2-ethylhydracrylic acid to increase abruptly in urine. On simplification of the spectrum of the latter compound to monoisotopic masses by the elimination of the portions of ion intensities due to natural abundances of heavy isotopes, this *R*-pathway metabolite was 3.8% unlabelled, 67.8% ^2H, and 28.4% $^2H_2$. Interpretation of its mass spectrum reveals that the label is to be found in its entirety in the hydroxymethyl

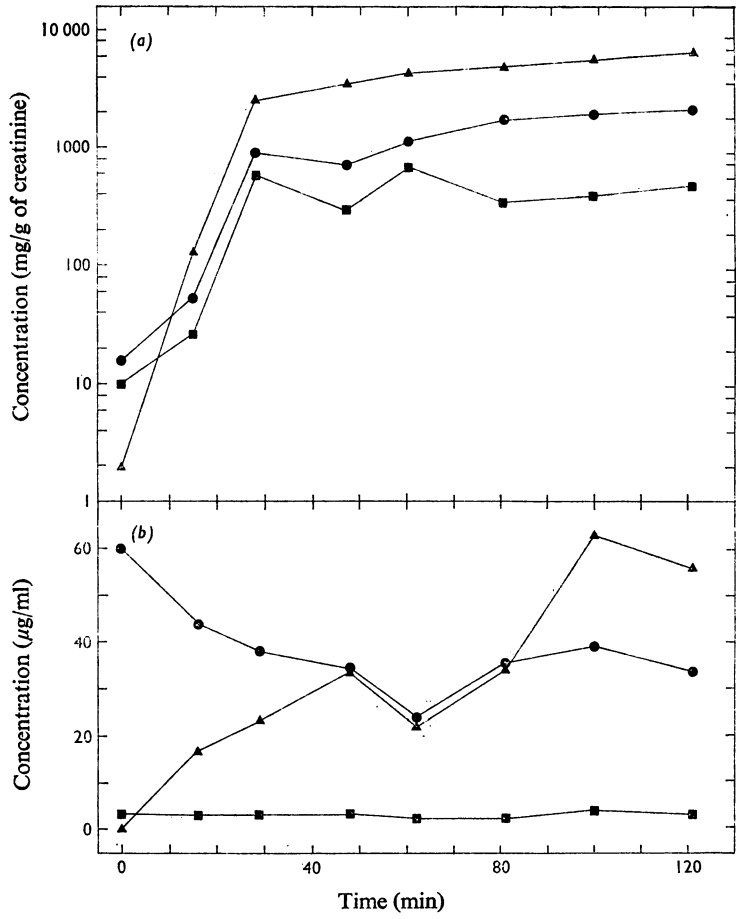

Fig. 2. *Time-course of the urine (a) and serum (b) concentrations of lactic acid (●), 3-hydroxybutyric acid (■) and 2-ethylhydracrylic acid (▲) in the dog after an intragastric load of racemic 2-methylbutyric acid*

2-Methyl-3-hydroxybutyric acid was not detectable at any time in the serum and showed an irregular excretion rate into the urine (maximum concentration 23 mg/g of creatinine). The apparent decrease in the serum acids at 62 min is a result of a sample-handling error.

Table 2. *Urinary excretion of organic acid in the rat after administration of fatty acid derivatives of isoleucine*

Injected acid was given intraperitoneally; urine collected for 24 h from paired animals each weighing 250–300 g. Control data are the range of excretion observed in three experiments. All data are excretion rates per single animal.

	Organic acids excreted (μmol/24 h per rat)				
	S Pathway		*R* Pathway		
Injected acid (mmol/kg)	2-Methyl-3-hydroxybutyric	Methylmalonic	2-Ethylhydracrylic	Ethylmalonic	Injected
None (control)	<0.3	<0.3	0.1–1.7	1.2–6.1	—
DL-2-Methyl[3,3,4,4,4-$^2H_5$]-butyric (1.63)	1.3*	2.3	18.2*	5.9*	15.0*
DL-2-[*Me*-$^2H_3$]Methylbutyric (1.64)	1.7*	3.0*	19.5*	6.4	13.3*
S(+)-2-Methylbutyric (1.64)	1.2	2.0	3.7	5.7	1.2
R(−)-2-Methylbutyric (1.64)	0.6	1.2	29.4	5.4	0.6
Tiglic (1.66)	2.2	6.6	2.4	3.9	15.7
2-Ethylacrylic (1.66)	0.7	4.0	63.3	3.9	<0.1

* ^2H substituted; see the text.

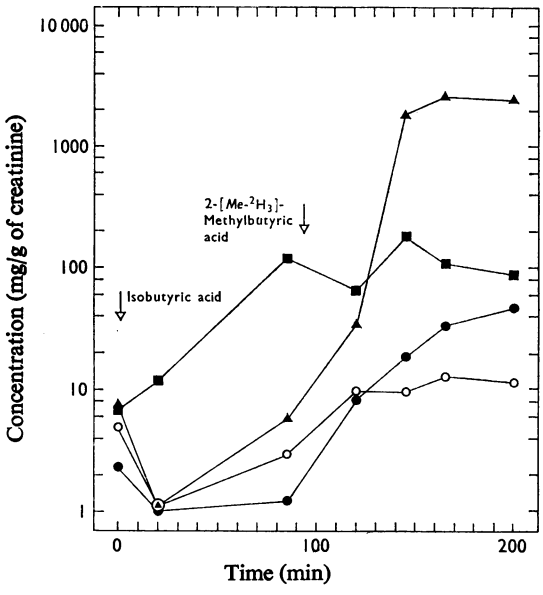

Fig. 3. *Time-course of the urine concentrations of* 2-*ethylhydracrylic acid* (▲), *ethylmalonic acid* (●), 2-*methyl*-3-*hydroxybutyric acid* (○) *and* 3-*hydroxyisobutyric acid* (■) *in the rabbit after gastric administrations of isobutyric acid and* 2-[*Me*-$^2H_3$]*methylbutyric acid at* 0 *and* 90 *min respectively*

For further details, see the text.

moiety (Mamer & Tjoa, 1975). The isotopic ratio ($^2H_1/^2H_2$) of the labelled metabolite was unaffected by varying the quantity of injected labelled precursor administered or by variation in the analytical procedure.

2-Methyl-3-hydroxybutyric acid and 2-methylacetoacetic acid were also labelled in excess of 90% with three ^2H atoms in their 2-methyl groups. Methylmalonic acid showed an unlabelled fraction increasing with time after administration of the labelled precursor. At no time were any of these last three acids unequivocally detected in the serum of the rabbit.

2-Methyl[3,3,4,4,4,-$^2H_5$]*butyric acid in the rats.* The gas chromatogram of acids excreted in the urine of rats injected intraperitoneally with 2-methyl[3,3,4,4,4-$^2H_5$]butyric acid is reproduced in Fig. 4. ^2H-labelling was evident in the following acidic metabolites: 2-hydroxy-2-methylbutyric acid, 94% $^2H_5$; 2-methyl-3-hydroxybutyric acid, 17.2% unlabelled, 1.4% $^2H_1$, 0.6% $^2H_2$, 18.2% $^2H_3$ and 62.6% $^2H_4$; 2-ethylhydracrylic acid, more than 97% $^2H_5$-substituted; ethylmalonic acid, 20.6% $^2H_5$, the remainder unlabelled; mesaconic acid, approx. 45% $^2H_5$, the remainder unlabelled; and 2-methylbutyrylglycine, completely $^2H_5$-labelled. The high mass portion of the mass spectrum taken at the top of the eluted ethylmalonate peak (peak no. 9, Fig. 4) is reproduced in Fig. 5, illustrating the presence of the ^2H label in ethylmalonic acid. The extent of labelling of bis(trimethylsilyl)ethylmalonate was determined by selected ion monitoring of *m/e* 266 and 261, the $M^{+\cdot}$-CH_3 ions of the $^2H_5$-substituted and unsubstituted esters respectively. The fragmentogram showed the labelled metabolite eluted about 4s ahead of the unlabelled metabolite under the gas-chromatographic conditions used.

Discussion

The metabolite 2-ethylhydracrylic acid (2-hydroxymethylbutyric acid) is a normal constituent of human and mammalian urine. Increased urinary excretion of this metabolite in man is found in patients with defective thiolase activity in the pathway for isoleucine oxidation; excretion is also increased two- to three-fold in methylmalonicaciduria, where the block is more distal in the catabolic outflow pathway, but permits accumulation of several more proximal metabolites. Excretion of 2-ethylhydracrylic acid is diminished in patients in whom a block in isoleucine (and alloisoleucine) oxidation occurs at the entry into the *S* and *R* pathways, as, for example, in maple-syrup-urine disease. 2-Ethylhydracrylic acid is excreted by man at an increased concentration after an isoleucine load (Mamer & Tjoa, 1974), under conditions where intestinal flora are suppressed by neomycin. Thus the *R* pathway appears to be a normal, if minor, component of isoleucine catabolism.

In β-oxothiolase deficiency 2-methyl-3-hydroxybutyrate may accumulate even more than 2-methylacetoacetate, perhaps through product inhibition of 3-hydroxyacyl-CoA dehydrogenase. Increased 2-ethylhydracrylic acid excretion in this disorder suggests that 3-hydroxyacyl-CoA dehydrogenase serves both the *R* and *S* pathways. In methylmalonicaciduria, where the block is more distal, the elevation of 2-ethylhydracrylic acid excretion is less pronounced.

The loading studies in animals with labelled or unlabelled racemic inputs of 2-methylbutyrate to both the *R* and *S* pathways reveal rapid and massive accumulations of 2-ethylhydracrylic acid. This finding again indicates endogenous formation of 2-ethylhydracrylic acid. Such a procedure, however, yields only modest increases in the urinary excretion of 2-methyl-3-hydroxybutyric acid, the *S*-pathway analogue of 2-ethylhydracrylic acid. Moreover, the *S*-pathway metabolite is not detectable at any time in serum under these conditions. Failure to accumulate the *S*-pathway metabolite (the *R*-pathway metabolite is, by contrast, retained at relatively high concentrations) suggests that rapid conversion into the corresponding 3-oxo metabolite and into propionyl-CoA is favoured by the *S* pathway, and that the analogous

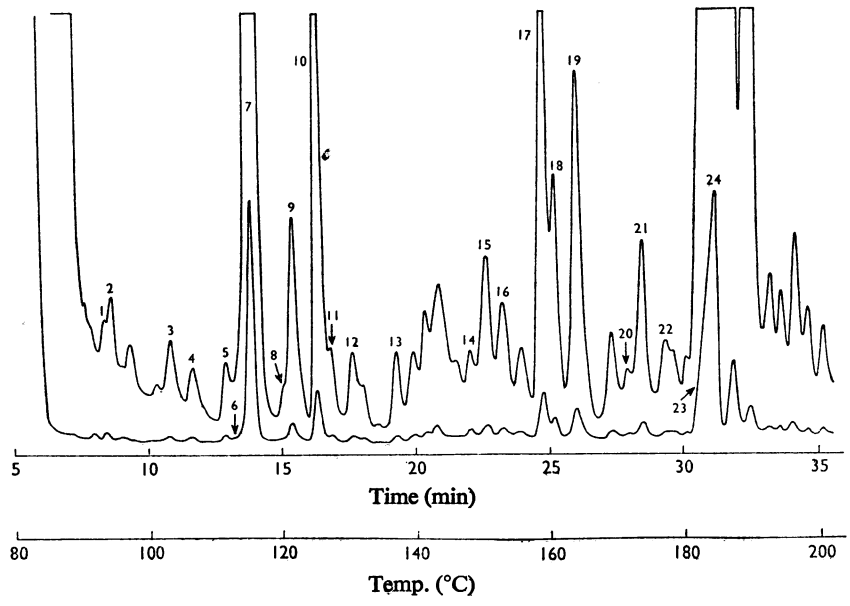

Fig. 4. *Gas chromatogram of acids in urine obtained after intraperitoneal administration of* 50*mg of racemic* 2*-methyl-*[3,3,4,4,4-$^2H_5$]*butyric acid to each of a pair of rats*

A glass column (2m×6mm) of 3% OV-101 on Chromosorb W HP was programmed to rise in temperature from 80°C at 4°C/min. Two separate tracings obtained with injections of different amounts of the same extract have been superimposed. The peaks are identified as the trimethylsilyl derivatives of the following acids: (1) lactic; (2) 2-hydroxyisobutyric; (3) 2-methyl-2-hydroxybutyric; (4) 3-hydroxyisobutyric; (5) 2-methyl-3-hydroxybutyric; (6) methylmalonic/3-hydroxyisovaleric, 3:1; (7) 2-ethylhydracrylic; (8) 2-ethyl-3-hydroxybutyric; (9) ethylmalonic; (10) succinic; (11) methylsuccinic; (12) fumaric; (13) mesaconic; (14) *N*-2-methylbutyrylglycine; (15) 3-methylhexenedioic; (16) 3-methyladipic; (17) unknown branched-chain C_8 dicarboxylic acid; (18) *m*-hydroxyphenylacetic and pimelic; (19) *p*-hydroxyphenylacetic; (20) suberic; (21) 3-(*m*-hydroxyphenyl)propionic; (22) aconitic; (23) and (24) hippuric, bis- and mono-trimethylsilyl derivatives respectively. Peaks 3, 5, 7, 9, 13 and 14 are labelled with ^2H.

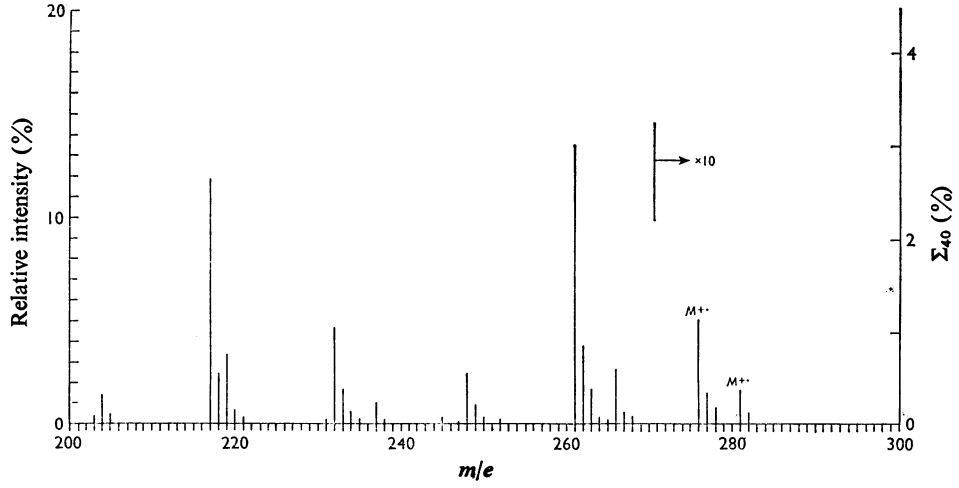

Fig. 5. *Upper mass range of the mass spectrum of the bis(trimethylsilyl) derivative of ethylmalonic acid excreted by rats after intraperitoneal injection of* 2*-methyl-*[3,3,4,4,4-$^2H_5$]*butyric acid*

The presence of two clearly distinguishable molecular ions at *m/e* 276 and 281, two $M^{+\cdot}$-CH_3 ions at *m/e* 261 and 266 and appropriate lighter fragment ions demonstrate the existence of $^2H_5$-substituted ethylmalonic acid in the urine. The spectrum is that of a mixture of ethylmalonic and authentic [*Et*-$^2H_5$]ethylmalonic trimethylsilyl derivatives.

oxidations via the *R* pathway are, by comparison, relatively inhibited beyond 2-ethylhydracrylic acid.

Evidence for different metabolic rates of the analogous 2-methyl-3-hydroxybutyric acid and 2-ethylhydracrylic acid (*S* and *R* metabolites respectively) is suggested by the failure of 2-ethylhydracrylic acid to maintain both ^2H substitutions on the hydroxymethyl moiety as might be expected by analogy with the following metabolic conversions:

Butyrate → crotonate → 3-hydroxybutyrate

Isobutyrate → methacrylate → 3-hydroxyisobutyrate

Isovalerate → 3-methylcrotonate → 3-hydroxyisopentanoate

The predominance of a $^2H_1$-labelled product after loading of the rabbit with racemic 2-[*Me*-$^2H_3$]-methylbutyrate is consistent with the enzyme-mediated exchange of one more ^2H atom than is required by simple stoicheiometry, and may be the result of a reversible oxidation step between 2-ethylhydracrylic acid and ethylmalonyl semialdehyde served by 3-hydroxyacyl-CoA dehydrogenase (Fig. 6). This postulate parallels the oxidation of 3-hydroxybutyric acid, 3-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid to acetoacetic acid, methylmalonyl semialdehyde and 2-methylacetoacetic acid in the catabolic pathways for straight-chain fatty acids, valine, and isoleucine (*S* pathway) respectively. Reduction of the aldehyde group back to the hydroxymethyl form would account for the loss of this second ^2H atom. Further and complete loss of the last ^2H label may be inhibited by the restriction of rotation of the bond between the hydroxymethyl group and the α-carbon atom in the bound substrate, which may deny this last ^2H accessibility to the enzyme, causing it to remain as the aldehydic hydrogen in the semialdehyde.

Further catabolism of ethylmalonyl semialdehyde to ethylmalonic acid is demonstrated by the finding that 20.6% of the ethylmalonic acid found in the rabbit urine bore five ^2H atoms. Butyryl-CoA may be an intermediate between the semialdehyde and the acid, by analogy with the terminal steps of valine oxidation recently illuminated by Tanaka *et al.* (1975). Butyryl-CoA would be formed by decarboxylation and CoA esterification of the semialdehyde, would retain the full $^2H_5$ label, and would recarboxylate to form ethylmalonate with the label intact. Propionyl-CoA carboxylase has been shown to serve this function (Hegre *et al.*, 1959).

Prior exogenous loading with unlabelled isobutyric acid in the rabbit appears not to interfere with the accumulation in urine of labelled 2-ethylhydracrylic acid and other labelled metabolites that can derive from a later loading of 2-[*Me*-$^2H_3$]methylbutyric acid.

Injection of the two *R*-pathway fatty acids initiated large increases in urinary excretion of 2-ethylhydracrylic acid while stimulating to a lesser degree methylmalonic acid and 2-methyl-3-hydroxybutyric acid in the *S* pathway. Conversely, administration of the *S* fatty acids precipitated a somewhat larger excretion of 2-methyl-3-hydroxybutyric acid and methylmalonic acid, and the response seen for 2-ethylhydracrylic acid was very much less than after injection of the *R* fatty acids. These observations suggest that, although oxidative decarboxylation of the *S* or the *R* 2-oxo acid forms the *S* or the *R*

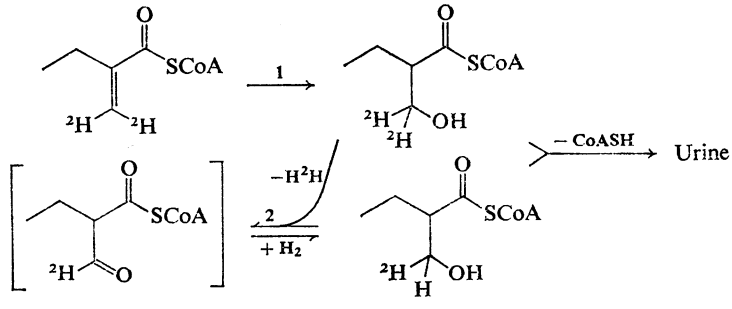

Fig. 6. *Proposed pathway to account for the predominance of* 2-[$^2H_1$]*ethylhydracrylic acid in the urine after gastric and intraperitoneal administration of* 2-[*Me*-$^2H_3$]*methylbutyric acid in the rabbit and rat*

Hydration of the twice-labelled ethylacrylate by enoyl-CoA hydratase (1) probably yields initially 2-ethylhydracrylate bearing two intact ^2H labels. Partial equilibration of one chirally specific ^2H label with aqueous protium may occur on reversible oxidation of the thioester with 3-hydroxyacyl-CoA dehydrogenase (2) before hydrolysis to the free acid and excretion.

enantiomer of 2-methylbutyric acid, which is then essentially committed to the *S* or *R* pathway respectively, there may be some interaction of metabolites in one pathway with the other under the conditions of our experiments *in vivo*.

S 2-Methylbutyric acid and tiglic acid appear to augment the excretion of methylmalonic acid in the mammal more effectively than the corresponding *R* metabolites. Methylmalonic acid is a product of the *S* pathway of isoleucine, formed from the carboxylation of propionyl-CoA derived both from 2-methylacetoacetyl-CoA and from methylmalonyl semialdehyde in the valine pathway (Tanaka *et al.*, 1975). Administration of fatty acids in the *R* pathway did not augment excretion of ethylmalonic acid to an equivalent degree, suggesting that the *R* pathway has minor significance *in vivo*. The rather low (20.6%) ^{2}H-labelled proportion of the ethylmalonate excreted by the rat after administration of 2-methyl[3,3,4,4,4-$^2H_5$]-butyric acid indicates the relative amounts of this metabolite that can be derived by this alternative pathway for isoleucine oxidation at high precursor concentration. This supports the view that catabolic steps beyond 2-ethylhydracrylic acid are attenuated relative to those that precede it and to the corresponding steps in the *S* pathway. We suggest that the unlabelled fraction of the ethylmalonate in urine in these experiments could in part be derived also from valine.

Formation of 2-ethylacrylic acid from 2-methylbutyrate is apparently poorly reversible under the conditions of our experiments. Injection of ethylacrylic acid in the rat did not cause the accumulation of 2-methylbutyrate, whereas the forward metabolite, 2-ethylhydracrylic acid, was greatly increased. Further, after injection of racemic 2-[*Me*-$^2H_3$]-methylbutyric acid, the large amount of free 2-methylbutyric acid recovered in the urine was as completely labelled with three ^{2}H atoms as the material injected. It would thus be unlikely that 2-ethylacrylate is formed initially with less than two ^{2}H atoms intact. The actual extent of labelling of this intermediate could not be determined, as it was never excreted into the urine in detectable amounts.

Elevated excretion of 2-methyl-3-hydroxybutyric acid, 2-methylacetoacetic acid and methylmalonic acid heavily labelled with three ^{2}H atoms in their 2-methyl groups after the injection of 2-[*Me*-$^2H_3$]-methylbutyric acid indicates that there is little endogenous dilution with the unlabelled metabolites in the rodent. In this sense the rodent experiments may reveal differences in flux of the pathway and the steady-state pool size of its intermediates compared with that of the *S* pathway in man, where accumulation of endogenous intermediates as far back as tiglic acid is evident in patients with thiolase deficiency (Keating *et al.*, 1972; Daum *et al.*, 1973).

Mesaconic acid and 2-methyl-2-hydroxybutyric acid containing ^{2}H labels were also found in urine in rabbit and rat injected with labelled 2-methylbutyric acid. Biochemical pathways producing these two acids do not, to our knowledge, use 2-methylbutyrate or its known *S*-pathway catabolites as substrates. The circumstances of their appearance bearing the label deserves further study.

Finally, the identification of labelled 2-methylacetoacetic acid in our experiments apparently provides the first direct support for the proposal of Robinson *et al.* (1956) that 2-methylacetoacetyl-CoA is an intermediate of isoleucine catabolism and a precursor of propionate. Until now, only the human disorder of 3-oxoacyl thiolase deficiency (Daum *et al.*, 1971; Keating *et al.*, 1972; Daum *et al.*, 1973; Hillman & Keating, 1974; Gompertz *et al.*, 1974) has yielded additional, but still indirect, evidence for this proposal. The modifications of 2-ethylhydracrylic acid excretion reported here indicate clearly, we believe, that a minor *R* pathway also serves the disposal of isoleucine (alloisoleucine) intermediates in the mammal.

We are grateful to Donna Zborowska-Sluis and Jacques Holman for technical assistance. These studies were supported in part by grants to O. A. M., G. K. and C. R. S. from the Medical Research Council of Canada and to C. R. S. from the Quebec Network of Genetic Medicine.

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